AWARD NUMBER: W81XWH-12-1-0395

TITLE: A Novel Mechanism for the Pathogenesis of Nonmelanoma Skin Cancer Resulting from Early Exposure to Ultraviolet Light

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REPORT DATE: September 2014

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
September 2014	Annual	1 Sept 2013 - 31 Aug 2014

4. TITLE AND SUBTITLE

A Novel Mechanism for the Pathogenesis of Nonmelanoma Skin Cancer Resulting from Early Exposure to Ultraviolet Light

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-12-1-0395

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

The Regents of the University of Minnesota
Minneapolis, MN 55455

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT We proposed that skin egress may be a characteristic of skin stem cells in response to ultraviolet light. Despite the poor breeding characteristics of our transgenic mice, we made 3 significant findings: 1) The first of two skin grafting studies was performed Aug. 14th-28th. FACS data were collected and analyzed. Practice surgeries were performed to get technique down. A repetition of this study will be performed on October 2nd. 2) A small pilot DMBA/TPA study was performed to assess whether keratinocytes migrated from the epidermis during the early stages of skin tumor promotion. Tissues were collected and analyzed. A larger experiment with additional mice will be performed this Fall. 3) We demonstrated using our K14EGFP/TOM transgenic mice that a low but significant number of K14-expressing cells are found in the bone marrow. The significance of these findings is that: 1) skin egress might be a characteristic of skin stem cells in response to ultraviolet light, and 2) bone marrow may be a long-lived reservoir of sunlight initiated stem cells that can repopulate the skin even years later upon damage caused by petrochemicals, skin wounding, or physical, chemical, or thermal damage to the skin.

15. SUBJECT TERMS
non-melanoma skin cancer, epidermal keratinocytes, stem cells, migration

16. SECURITY CLASSIFICATION OF: U			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			19b. TELEPHONE NUMBER *(include area code)*
Unclassified	Unclassified	Unclassified	UU	21	USAMRMC

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INTRODUCTION:

The epidermal layer of the skin is composed largely of cells called keratinocytes. Keratinocytes in the basal layer are organized into subpopulations based on their proliferative nature and include stem cells (relatively rare) and transit amplifying cells (comprise most of the proliferating cells). When a stem cell divides, one daughter usually remains a stem cell while the other daughter gives rise to transit amplifying cells with limited proliferative potential. Upon completion of their divisions, transit amplifying cells undergo an orderly maturation process called terminal differentiation that includes their outward displacement through the suprabasal layers, production of high molecular weight keratins, loss of their nuclei, and formation of an impervious outer structure called the cornified envelope. This process is exceptionally orderly and maintains the normal thickness and cellularity, and the normal functions of the epidermis throughout life. This proposal focuses on the stem cells of the hair follicles because they not only serve as a reservoir of epidermal cells, they also possess remarkable regenerative potential and are known to be able to reconstitute a graft, to heal wounds, and even to give rise to non-melanoma skin cancer (1). Therefore, identification of stem cell behavioral characteristics and responses are critical problems in cutaneous biology. We proposed here the novel concept that skin egress, entering the circulation, and traveling throughout the body may be a new behavior of epidermal stem cells. We proposed that sunburn following exposure to sunlight has the capacity to make skin stem cells migrate. In this Discovery award we challenge the existing paradigm of skin cells in response to sunburn. We address the following question: Do hair follicle stem cells migrate from the skin following sunburn as a consequence of ultraviolet light induced inflammation? Our hypothesis is that sunburn makes the hair follicles stem cells leave the skin and enter the blood circulation, and home to the bone marrow. Therefore, we proposed in this pilot study to test the first axiom: that CD34 positive keratinocyte stem cells have the capacity for extracutaneous migration. We will accomplish this with a novel application of standard in vitro assays together with the use of two transgenic mice that are already available including two UV experiments, and a skin grafting experiment. The rationale for the in vitro experiment was that the cellular and molecular players involved may be identified, isolated, and manipulated. The rationale for the in vivo experiments is that the cellular players also may be identified and isolated and explored systemically under their native conditions.

BODY:

NOTE CONCERNING THE REVISED STATEMENT OF WORK 10APR2014:

We made four changes to our originally proposed Statement of Work. The first three changes involved mouse strains only and no change in the experimental procedures. The fourth change involved a procedure where we add a better-characterized and more-timely model to Specific Aim 2.

First, we substituted K15EGFP/TOM female mice (bred from K15CrePR1 x ROSAmTomato/mEGFP reporter mice) for the K15EGFP mice that are no longer available (The K15EGFP strain was frozen down in the time between submission of our proposal and its award.) Second, we added K14EGFP/TOM mice (bred from K14CRE x ROSAmTomato/mEGFP reporter mice) as a control for the K15EGFP/TOM mice because this K15 promoter is known to be leaky, and because the K14Cre mouse should label robustly all proliferating epidermal basal cells rather than the progeny of the K15-expressing hair follicle stem cells alone. Third, we added an additional 12 UBC/EGFP mice as "green-only" controls for our originally proposed FACS experiments because we had not proposed sufficient "green-only" controls for these experiments. Lastly, we proposed two DMBA/TPA experiments to supplement the UV experiment in Specific Aim 2 (new Tasks 7 and 8) because we realized that we could obtain a definitive and more timely answer to our question with this well-characterized chemical model of skin carcinogenesis than with the UV carcinogenesis model the early stages of which needed additional characterization which is outside the scope of this project.

We note that even though our K14EGFP/TOM experiments are behind schedule due to the poor breeding of these mice, we expect to be able to finish all experiments with the K14EGFP/TOM mice as planned in the coming unfunded year; however, we do not expect to be able to complete the experiments with the K15EGFP/TOM mice proposed in the revised SOW due to poor breeding of the mice, and lack of funds.

Changes to our originally proposed Statement of Work below are given in italics.

Specific Aim 1, Task 3 (5 mice). Epidermal keratinocytes harvested from individual *K15EGFP/TOM* and *K14EGFP/TOM* mice were placed in the Matrigel invasion assay as summarized in Experiment 1. In this experiment we will specifically focus on the percentage of the *K15EGFP/TOM* and *K14EGFP/TOM* immunoreactive cells. We have continued to pursue the migration assays with SDF1alpha and Hmgb1 together with their inhibitors using the new Millipore invasion assay kit, but the results were unsatisfactory. We have returned to the original Matrigel invasion assays.

MAJOR OBJECTIVES FOR AIM 2. The focus of this aim is was determine whether hair follicle stem cells or their progeny migrate from the skin during ultraviolet light induced inflammation and carcinogenesis. **General Approach.** We will establish skin grafts of *K14EGFP/TOM* enriched and depleted subpopulations, and quantify by FACS, fluorescent keratinocytes in the dermis, blood, bone marrow and other tissues.

MAJOR TASKS FOR SPECIFIC AIM 2

Specific Aim 2, Task 5 (20 female *K14EGFP/TOM* and 20 wild-type littermates). In preparation for Specific Aim 2, Task 5 we examined in Year 01 keratinocytes, blood, and bone marrow by FACS to determine whether we could detect keratin 14 immunoreactivity in blood and bone marrow. We had demonstrated that the percentage of keratin 14 expressing cells is normally quite low in both blood and bone marrow, but increases considerably in response to solar UV treatment. We still do not yet know whether this UV-dependent increase is a consequence of keratinocyte egress from the skin; however, we also had the opportunity to obtain bone marrow from euthanized mice in ongoing tumor experiments from other investigators at the Hormel Institute, and we looked for keratin 14 immunoreactive cells in blood and bone marrow by light microscopy of smears of nucleated cells. Although we still need more experience to perfect this experimental method, we were able to confirm the presence of keratin 14 immunoreactive cells in both blood and bone marrow. To investigate further this novel observation, we performed qPCR on freshly harvested epidermal keratinocytes, and on mononuclear cells from blood and bone marrow. As expected, both keratins 14 and 15 were detected in epidermal keratinocytes. However, we also detected them in blood and bone marrow from C57BL/6 mice, and keratin 14 only in the blood and bone marrow of BALB/c mice. Therefore, we have confirmed the presence of epidermal cytokeratin 14 by three different methods, by FACS, by immunofluorescence microscopy, and by qPCR. In Year 02, we confirmed these results and extended them with our K14EGFP/TOM mice. We found that by using this transgenic model, there were green EGFP fluorescent cells present in blood and bone marrow. This is a highly significant and exciting finding because the transgenic model did not depend upon immunostaining or qPCR. **Figure 1a** demonstrates that the number of "green" cells in the bone marrow of K14EGFP/TOM (Hybrid) mice increases as the number of counted cells increases. **Figure 1b** summarizes typical FACS plots from this experiment demonstrating positive green counts in bone marrow from the K14EGFP/TOM hybrid mice but not in the negative control.

Specific Aim 2, Task 6 (15 female *K14EGFP/TOM* mice and 15 female wild-type littermates, 30 female SCID mice). In this experiment epidermal keratinocytes were harvested from *K14EGFP/TOM* female mice and from wild-type littermates. For the skin reconstitution assay, keratinocytes were surgically implanted onto a 1 cm diameter area of dorsal fascia using a silicone chamber initially to contain the cells. The grafts were replaced on SCID female mice. These grafts stabilized 1 week following implantation. All surgical procedures were conducted on anesthetized mice in a laminar flow hood with sterile technique according to procedures of the

University of Minnesota Research Animal Resources. Groups of 5 grafts were harvested at 4 day, 7 day, and 14 day time points. "Green" EGFP fluorescent keratinocytes were quantified by FACS in blood and bone marrow. In the grafts, we observed a small amount of epithelial tissue that had formed by 7 days post-surgery, and robust engraftment of epithelial tissue at 14 days. Immunohistochemistry has not yet been performed on the grafts themselves. In summary, we did not find EGFP fluorescent cells in bone marrow from any of the SCID mice. Some green cells were seen in blood from both SCID mice that received the CRE-negative control cells. Some green cells were observed in blood of SCID mice receiving hybrid cells. We cannot accurately interpret the results from this experiment due to a "tail-like" artifact in our FACS plots that we believe might be due to cells damaged in the lysing process to remove red blood cells. We have modified our lysing process and the "tail" has disappeared. Graphs documenting this experiment are shown in **Tables 1** (blood) and **2** (bone marrow). These data are not sufficient to support nor refute our hypothesis. This experiment will be repeated on October 2nd through 20th.

Specific Aim 2, Task 7 (5 female K14EGFP/TOM mice and 5 female wild-type littermates, 5 female C57Bl/6). *In this experiment, K14EGFP/TOM, wild-type littermates, and B6 mice received one dose of DMBA followed by three applications of TPA or acetone weekly for two weeks. After two weeks of TPA promotion, all mice were euthanized. "Green" EGFP reactive keratinocytes were quantified by FACS of blood and bone marrow.* A partial analysis of photomicrographs is summarized in **Figures 2 through 6.** Figures 2 and 3 summarize "green" cells in bone marrow, with Figure 2 being the broad picture, and Figure 3 zooming in on the low, but reproducible, level of "green" cells in bone marrow in response to DMBA and TPA Treatment. Similarly, **Figures 4 through 6** summarize cell counts in blood, with Figures 4 and 5 being the broader picture and Figure 6 zooming in on the low, but reproducible, level of green cells in the blood. Basically, we observed more EGFP positive and EGFP/TOM double positive cells in blood than in bone marrow. Unexpectedly, there were fewer cells in the DMBA/TPA treated group than in the control treated groups (ACE/TPA, DMBA/ACE/ and ACE/ACE). The interpretation of this is not readily apparent, and we expect that we will need to investigate the time-course during TPA promotion in greater detail. Nevertheless, we can conclude from this experiment that DMBA/TPA treatment of the skin results in an increase in K14EGFP positive cells in the blood and bone marrow.

Specific Aim 2, Task 8 (4 male K14EGFP/TOM mice, 4 male UBC/GFP mice, 4 male C57Bl/6 mice and 27 female C57Bl/6; 4 male K14EGFP/TOM mice, 4 male UBC/GFP, 4 male C57Bl/6 and 27 female C57Bl/6 mice). *In this experiment female B6 bone marrow transplant (BMT) recipient mice received bone marrow transplants from male K15EGFP/TOM, UBC/GFP, or B6 mice that had been treated with one dose of DMBA followed by three applications of TPA or acetone weekly for two weeks. After four weeks of BMT engraftment, female BMT recipients received three applications of TPA or acetone weekly for two weeks then all mice were euthanized. "Green" EGFP reactive keratinocytes were quantified by fluorescence microscopy or EGFP immunostaining in the dermis, blood, lymph nodes, and bone marrow as described. Blood and bone marrow samples were analyzed with FACS to determine differences in populations of "green" EGFP expressing cells after different treatments of DMBA/TPA. For FACS analysis, UBC/EGFP control mice are needed to calibrate the flow cytometer.* **Figure 7a** shows an increased number of EGFP immunoreactive bone marrow derived cells migrating to the skin relative to controls early in the course of TPA tumor promotion**.** These data do not demonstrate the return of egressed keratinocyte from bone marrow back to the skin; however, the sample size was too small and an insufficient number of cells was counted. **Figure 7b** summarizes the treatment of the mice. The remainder of the tissue blocks will be processed in the coming weeks.

MAJOR MILESTONES FOR AIM 2. In the first experiment, if our hypothesis were correct, then we would expect to see a UV dose-dependent increase in the number of skin-derived EGFP-reactive keratinocytes in non-cutaneous tissues that we indeed do observe. If our hypothesis were partially correct, we should find metastatic cells during the later stages of skin tumor progression. Similarly, in the second experiment, we would find EGFP immunoreactive keratinocytes in blood and bone marrow following establishment of the skin grafts. The

results of our first experiment were equivocal, and must be confirmed. Substantial analysis of tissue samples and will follow through next year, as the poor breeding of the transgenic mice thwarted our efforts for a timely conclusion of this project.

KEY RESEARCH ACCOMPLISHMENTS FOR YEAR 02:

- The first of two skin grafting studies for the DOD grant was performed. This experiment was performed on Aug 14th and concluded on Aug 28th. FACS data were collected and analyzed shortly thereafter. Practice surgeries were performed to get technique down and to ensure that all supplies we need were ordered. A repetition of this study will be performed on October 2nd.
- A small pilot DMBA/TPA study was performed to assess whether keratinocytes migrated from the epidermis during the early stages of skin tumor promotion. Tissues were collected and analyzed. A larger experiment with additional mice will be performed this Fall.
- We demonstrated using our K14EGFP/TOM transgenic mice that a low but significant number of K14-expressing cells are found in the bone marrow in response to DMBA/TPA treatment of the skin.

REPORTABLE OUTCOMES YEAR 02:

- No reportable outcomes were made in Year 02.
- Submission of a letter to the editor of Journal of Investigative Dermatology and a Manuscript is now anticipated by the Spring of 2015.
- We anticipate submitting an R01 proposal on the subject of this DOD award upon completion of our work in the Summer of 2015.
- We expected to make a presentation of this research at the International Skin Carcinogenesis Conference in June of 2014 but had to decline due to a death in the PI's family. Tentative plans are in progress to present this work at the Gordon Research Conference of Epithelialization and Keratinization in the summer of 2015.

CONCLUSIONS FROM OUR RESEARCH IN YEAR 02:

We conclude that the data presented herein continue to support our novel hypothesis that (mouse) skin keratinocytes can leave the cutaneous epithelium and enter the blood and bone marrow. Should these findings hold true in the coming months upon analysis of the remaining data, the medical implications would be that the bone marrow could be a reservoir of transformed keratinocytes with the potential to migrate back to the skin as cancer initiating cells upon further damage to the skin. The implications for the US Military would be 1) the need to prevent the first damaging exposure to the skin, and 2) the need to prevent the re-recruitment of the initiated keratinocytes back to the skin by manipulating the cytokine pathways used (possibly SDF1alpha or HMGB1?) upon further damage to the skin such as by combat wounds. Further, if our findings are substantiated by the in vivo studies currently in progress and by complementary findings in humans that we will propose in an R01 next year, they would suggest that the number of keratin 14-expressing cells in the blood could provide a simple test for persons at risk for developing non-melanoma skin cancer.

REFERENCES:

1. Li, S., Park, H., Trempus, C.S., Gordon, D., Liu, Y., Cotsarelis, G., and **Morris, R.J.** Keratin 15 expressing stem cells from the hair follicles contribute to papilloma development in the mouse. Mol. Carcinogenesis 2012, Epub ahead of print, Li, Park, and Trempus share equally as first author.

2. Tamai, K., Yamazaki, T., Chino, T., Ishii, M., Otsuru, S., Kikuchi, Y., Iinima, S., Kaga, K., Nimura, K., Shimbo, T., Umegaki, N., Katayama, I., Miyazaki, J., Takeda, J., McGrath, J.A., Uitto, J., and Kaneda, Y. PDGFR1 alpha-positive cells in bone marrow are mobilized by high mobility group box 1 (HMGB1) to regenerate injured epithelia. Proc. Natl. Acad. Sci. USA 108: 6609-6614, 2011.

APPENDIX:

The Figures representing the principal findings during Year 02 follow here in order as one page per figure together with descriptive legends. Thirteen (13) pages of Appendix Figures follow.

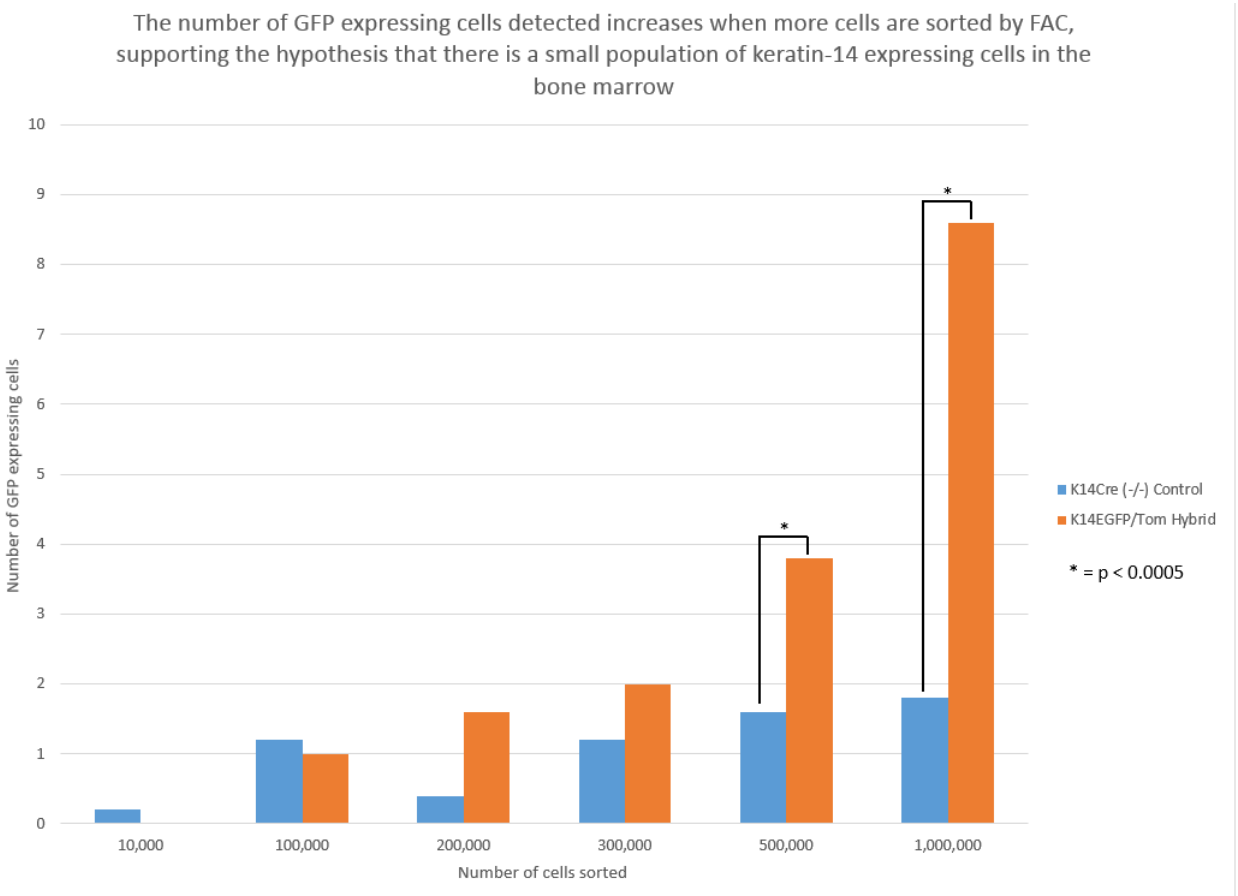
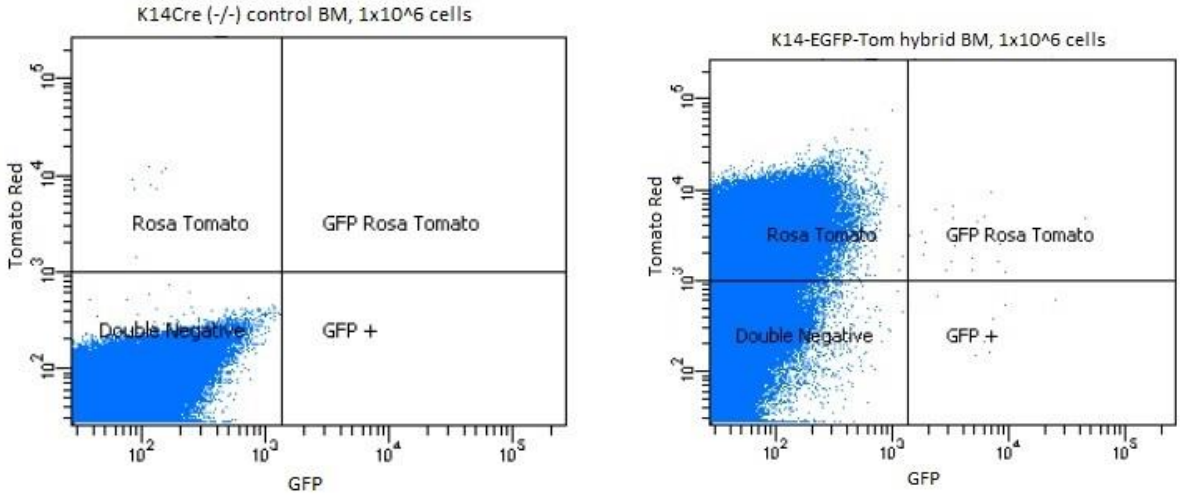


Figure 1a. Fluorescent activated cell sorting (FACS) shows increased detection of GFP expressing keratin-14 cells in the bone marrow as more cells are sorted. In the K14-EGFP-Tom mice, K14 expressing cells naturally fluoresce “green” whereas all other cells naturally fluoresce “red”. The average number of K14-EGFP fluorescent labeled cells in the bone marrow of K14-EGFP-Tom hybrid increased as a larger number of cells were sorted in a sample. A two-tailed T-test shows statistical significance ($p < 0.0005$) between the K14-EGFP-Tom cells and the negative control cells when 500,000 or 1,000,000 cells are sorted.



Tube: K14-EGFP-Tom hybrid BM, 1x10^6 cells

Population	#Events	%Parent	%Total
All Events	1,240,287	####	100.0
P1	1,044,693	84.2	84.2
Live Cell (7AAD-)	1,000,000	95.7	80.6
Rosa Tomato	417,935	41.8	33.7
GFP Rosa Tomato	19	0.0	0.0
Double Negative	582,039	58.2	46.9
GFP +	7	0.0	0.0

Figure 1b. FACS dot plots and event table demonstrating the difference in cell populations based on fluorescent signal. The K14-EGFP-Tom hybrid mice show a large population of cells that fluoresce with Tomato Red and few cells that fluoresce with GFP only or GFP/Tomato Red double positive relative to K14Cre negative control mice. This K14-EGFP-Tom hybrid strain is designed to have keratin 14 expressing cells fluoresce “green” while all other cells fluoresce “red”. Sorting for each of the event cut-offs shown in Figure 1a (10,000 cells, 100,000 cells, and so on) were performed but omitted for the sake of brevity. Sorts of the same event cut-offs were performed on multiple occasions and show similar dot plots and event numbers as depicted here.

Table 1: Inconclusive results regarding the migration of keratin 14 GFP expressing cells found in the blood of SCID mice after receiving keratinocyte skin grafts. The GFP positive signal in the test group detected from FACS is mostly part of a tail that has believed to be due to damaged cells.

Ms #	Treatment	Date Euth	# cells	% Double Negative	% GFP	% TomRed	% Double Positive
3	SCID-K14Cre Negative Control	1d	100,000	100	0	0	0
			500,000	100	0	0	0
			700,000	100	0	0	0
5	SCID-K14Cre	4d	100,000	100	0	0	0
			500,000	100	0	0	0
			1,000,000				
6	SCID-K14Cre	4d	100,000	100	0	0	0
			500,000	100	0	0	0
			1,000,000				
1	SCID-K14Cre	7d	100,000	100	0	0	0
			500,000	100	0	0	0
			1,000,000				
4	SCID-K14Cre	7d	100,000	99.9	0.1	0	0
			500,000	99.9	0.1	0	0
			1,000,000				
2	SCID-K14Cre	7d	100,000	99.2	0.8	0	0
			500,000	99.2	0.8	0	0
			1,000,000				
7	SCID-K14Cre	14d	100,000	100	0	0	0
			500,000	100	0	0	0
			1,000,000				
8	SCID-K14Cre	14d	100,000	100	0	0	0
			500,000	100	0	0	0
			1,000,000				
10	**SCID-K14GFP Test Group**	**4d**	**100,000**	**100**	**0**	**0**	**0**
			500,000				
			1,000,000				
11	**SCID-K14GFP**	**4d**	**100,000**	**100**	**0**	**0**	**0**
			500,000	**100**	**0**	**0**	**0**
			1,000,000				
12	**SCID-K14GFP**	**7d**	**100,000**	**99.3**	**0.7**	**0**	**0**
			500,000				
			1,000,000				
13	**SCID-K14GFP**	**7d**	**100,000**	**99.1**	**0.9**	**0**	**0**
			500,000	**99**	**1**	**0**	**0**
			1,000,000				
14	**SCID-K14GFP**	**14d**	**100,000**	**100**	**0**	**0**	**0**
			500,000	**100**	**0**	**0**	**0**

15	**SCID-K14GFP**	**14d**	**700,000 100,000 500,000 1,000,000**	**99.5**	**0.5**	**0**	**0**
	UBC-GFP Green +Control	4d	100,000 500,000 1,000,000	8 7.9	92 92.1	0 0	0 0
	ROSA-TOM Red + Control	4d	100,000 500,000 1,000,000	99.9	0.1	0	0
	UBC-GFP	7d	100,000 500,000 1,000,000	5.6	94.4	0	0
	ROSA-TOM	7d	100,000 500,000 1,000,000	17.9	0	82.1	0
	UBC-GFP	14d	100,000 500,000 1,000,000	11.1	0	88.9	0
	ROSA-TOM	14d	100,000 500,000 1,000,000	17.5	0	82.5	0
	Negative control	14d	100,000 500,000 1,000,000	100	0	0	0

Table 2: No migration of keratin 14 GFP expressing cells in the bone marrow of SCID mice after receiving keratinocyte skin grafts.

Ms #	Treatment	Date Euth	# cells	% Double Negative	% GFP	% TomRed	% Double Positive
3	SCID-K14Cre Negative control	1d	100,000	100	0	0	0
			500,000	100	0	0	0
			700,000	100	0	0	0
5	SCID-K14Cre	4d	100,000	100	0	0	0
			500,000	100	0	0	0
			1,000,000	100	0	0	0
6	SCID-K14Cre	4d	100,000	100	0	0	0
			500,000	100	0	0	0
			1,000,000	100	0	0	0
1	SCID-K14Cre	7d	100,000	100	0	0	0
			500,000	100	0	0	0
			1,000,000	100	0	0	0
4	SCID-K14Cre	7d	100,000	100	0	0	0
			500,000	100	0	0	0
			1,000,000	100	0	0	0
2	SCID-K14Cre	7d	100,000	100	0	0	0
			500,000	100	0	0	0
			1,000,000				
7	SCID-K14Cre	14d	100,000	100	0	0	0
			500,000	100	0	0	0
			1,000,000	100	0	0	0
8	SCID-K14Cre	14d	100,000	100	0	0	0
			500,000	100	0	0	0
			1,000,000	100	0	0	0
10	**SCID-K14GFP Test Group**	**4d**	**100,000**	**100**	**0**	**0**	**0**
			500,000	**100**	**0**	**0**	**0**
			1,000,000	**100**	**0**	**0**	**0**
11	**SCID-K14GFP**	**4d**	**100,000**	**100**	**0**	**0**	**0**
			500,000	**100**	**0**	**0**	**0**
			1,000,000	**100**	**0**	**0**	**0**
12	**SCID-K14GFP**	**7d**	**100,000**	**100**	**0**	**0**	**0**
			500,000	**100**	**0**	**0**	**0**
			700,000	**100**	**0**	**0**	**0**
13	**SCID-K14GFP**	**7d**	**100,000**	**100**	**0**	**0**	**0**
			500,000	**100**	**0**	**0**	**0**

			1,000,000	**100**	**0**	**0**	**0**
14	**SCID-K14GFP**	**14d**	**100,000**	**100**	**0**	**0**	**0**
			500,000	**100**	**0**	**0**	**0**
			1,000,000	**100**	**0**	**0**	**0**
15	**SCID-K14GFP**	**14d**	**100,000**	**100**	**0**	**0**	**0**
			500,000	**100**	**0**	**0**	**0**
			1,000,000	**100**	**0**	**0**	**0**
	UBC-GFP Green+ Control	4d	100,000	8.1	91.9	0	0
			500,000	7.7	92.3	0	0
			1,000,000	7.2	92.8	0	0
	ROSA-TOM Red + Control	4d	100,000	100	0	0	0
			500,000				
			1,000,000				
	UBC-GFP	7d	100,000	18.6	81.4	0	0
			500,000				
			1,000,000				
	ROSA-TOM	7d	100,000	16.7	0	83.3	0
			500,000				
			1,000,000				
	UBC-GFP	14d	100,000	19.5	0	80.5	0
			500,000				
			1,000,000				
	ROSA-TOM	14d	100,000	9.8	0	90.2	0
			500,000				
			1,000,000				
	Negative control	14d	100,000	100	0	0	0
			500,000				
			1,000,000				

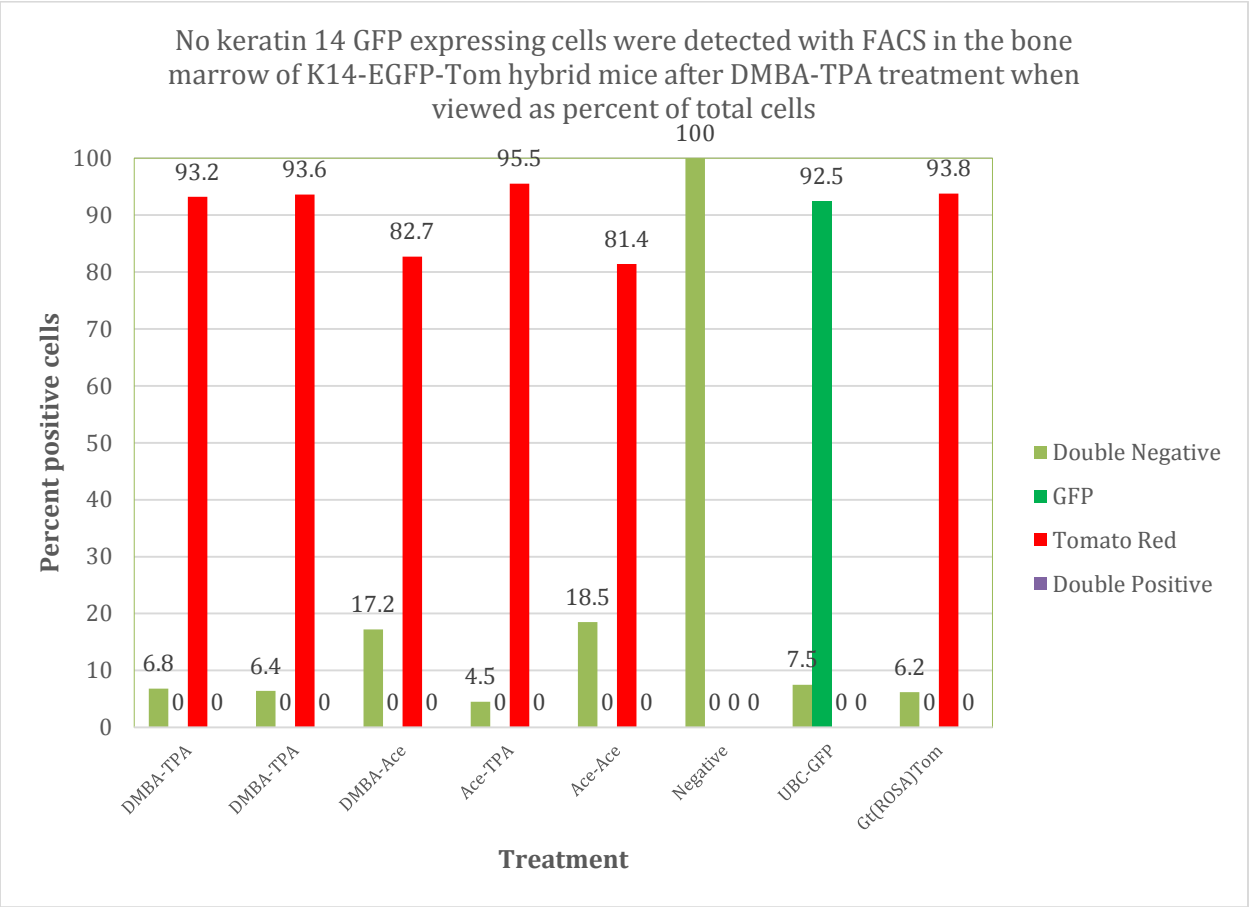


Figure 2. Fluorescent activated cell sorting (FACS) shows no K14-GFP expressing cells in the bone marrow of K14-EGFP-Tom hybrid mice after receiving DMBA and TPA treatment. The percent distribution of K14-EGFP fluorescent labeled cells in the bone marrow of K14-EGFP-Tom hybrid mice after 1x initiation with DMBA and 6x promotion treatments with TPA. Acetone (Ace) was used as a control. DMBA and TPA treatments were also performed on K14Cre and B6 mice as negative controls (data omitted). No treatment was performed on the Negative, UBC-GFP, or Gt(ROSA)Tom mice as these were only used as FACS controls. In the K14-EGFP-Tom mice, K14 expressing cells naturally fluoresce “green” whereas all other cells naturally fluoresce “red”. When analyzing the bone marrow samples by percent positive cells, there are no K14 “green” cells among any of the conditions.

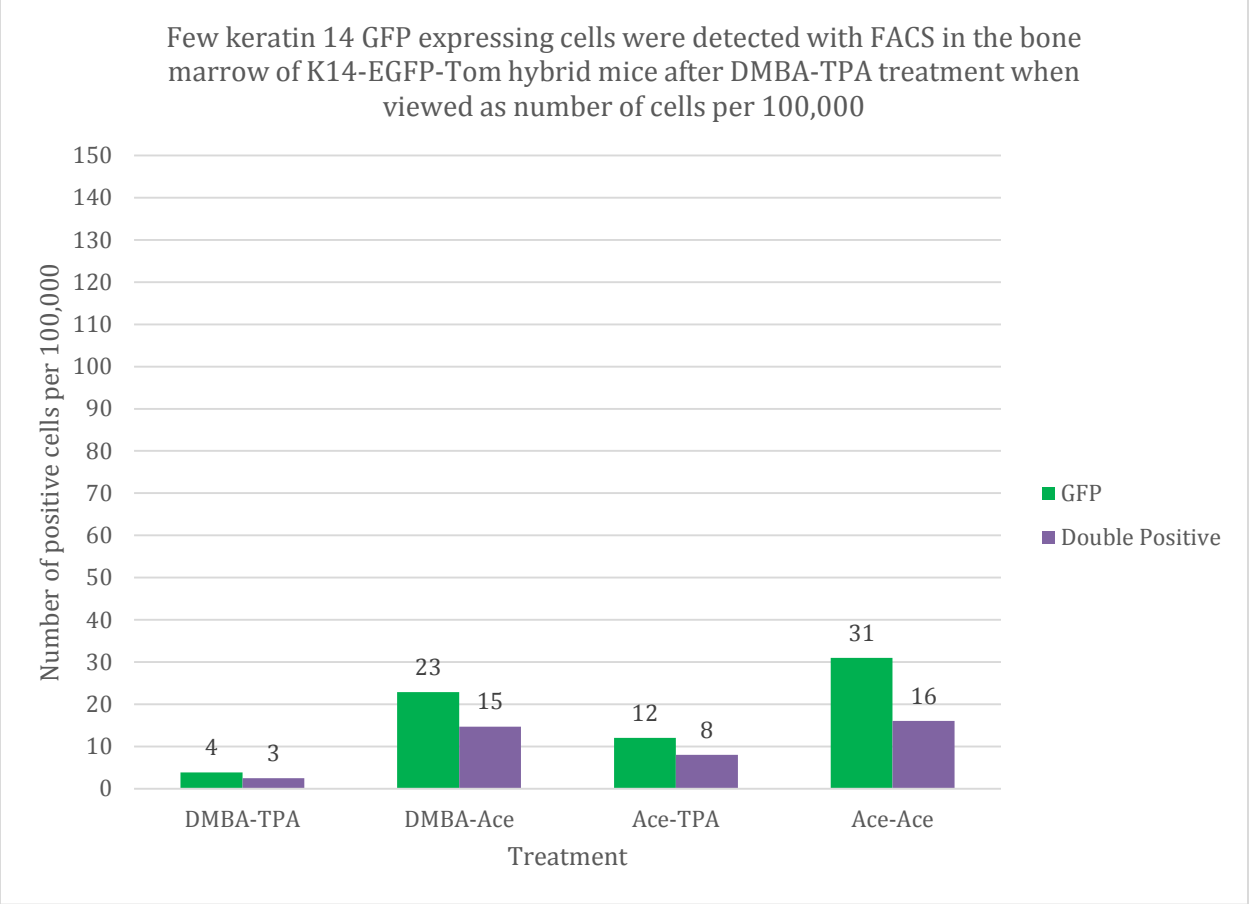


Figure 3. Fluorescent activated cell sorting (FACS) shows slight detection of K14-GFP expressing cells in the bone marrow of K14-EGFP-Tom hybrid mice after receiving DMBA and TPA treatment cells. The number of positive K14-EGFP fluorescent labeled cells per 100,000 sorted in the bone marrow of K14-EGFP-Tom hybrid mice after 1x initiation with DMBA and 6x promotion treatments with TPA. Acetone (Ace) was used as a control. DMBA and TPA treatments were also performed on K14Cre and B6 mice as negative controls (data omitted). In the K14-EGFP-Tom mice, K14 expressing cells naturally fluoresce “green” whereas all other cells naturally fluoresce “red”. When analyzing the bone marrow samples by number of GFP positive or GFP/TomatoRed double positive cells, there are fewer GFP positive and GFP/TomatoRed double positive cells in the DMBA-TPA treatment groups than in the various controls.

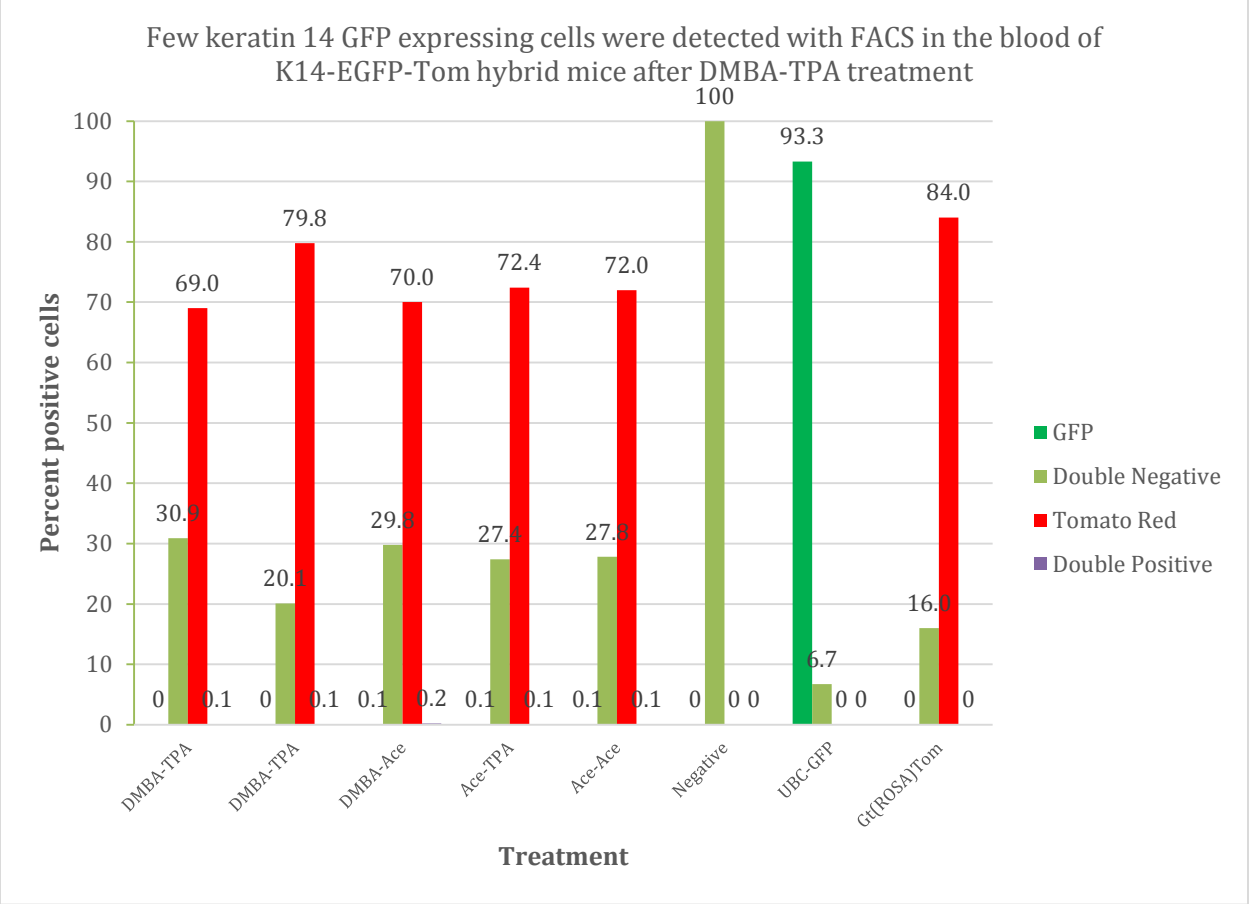


Figure 4. Fluorescent activated cell sorting (FACS) shows slight detection of K14-GFP expressing cells in the blood of K14-EGFP-Tom hybrid mice after receiving DMBA and TPA treatment (data displayed as 0-100% total cells). The percent distribution of K14-EGFP fluorescently labeled cells in the blood of K14-EGFP-Tom hybrid mice after 1x initiation with DMBA and 6x promotion treatments with TPA. Acetone (Ace) was used as a control. DMBA and TPA treatments were also performed on K14Cre and B6 mice as negative controls (data omitted). No treatment was performed on the Negative, UBC-GFP, or Gt(ROSA)Tom mice as these were only used as FACS controls. In the K14-EGFP-Tom mice, K14 expressing cells naturally fluoresce “green” whereas all other cells naturally fluoresce “red”. When analyzing the blood samples by percent positive cells, 0.1% of cells are GFP positive or GFP/TomatoRed double positive except for the DMBA-TPA treated mice in which no cells were GFP positive.

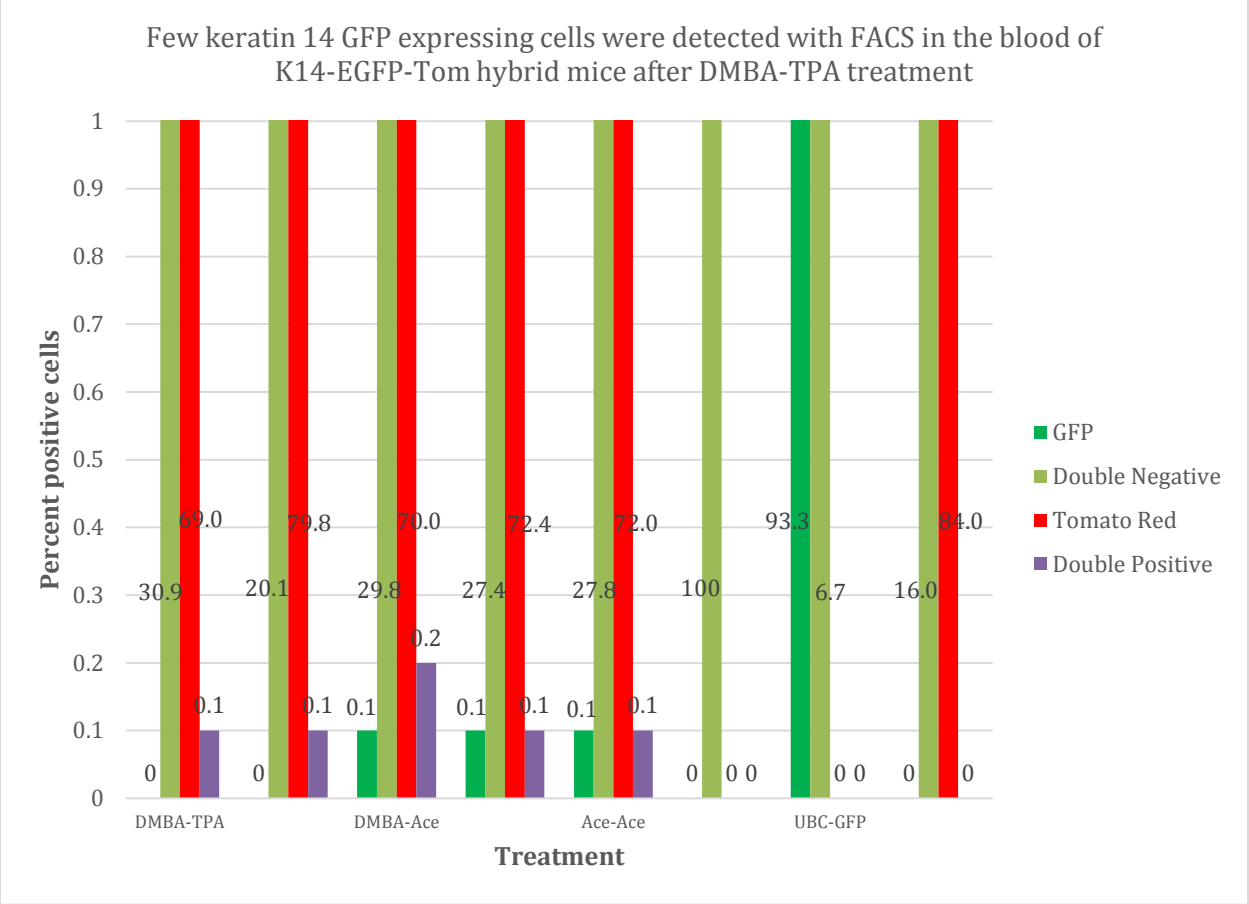


Figure 5. Fluorescent activated cell sorting (FACS) shows no K14-GFP expressing cells and slight expression of GFP/TomatoRed double positive cells in the blood of K14-EGFP-Tom hybrid mice after receiving DMBA and TPA treatment (data displayed as 0-1% of total cells). The percent distribution of K14-EGFP fluorescent labeled cells in the blood of K14-EGFP-Tom hybrid mice after 1x initiation with DMBA and 6x promotion treatments with TPA. Acetone (Ace) was used as a control. DMBA and TPA treatments were also performed on K14Cre and B6 mice as negative controls (data omitted). No treatment was performed on the Negative, UBC-GFP, or Gt(ROSA)Tom mice as these were only used as FACS controls. In the K14-EGFP-Tom mice, K14 expressing cells naturally fluoresce “green” whereas all other cells naturally fluoresce “red”. When analyzing the blood samples by percent positive cells, 0.1% of cells are GFP positive or GFP/TomatoRed double positive except for the DMBA-TPA treated mice in which no cells were GFP positive.

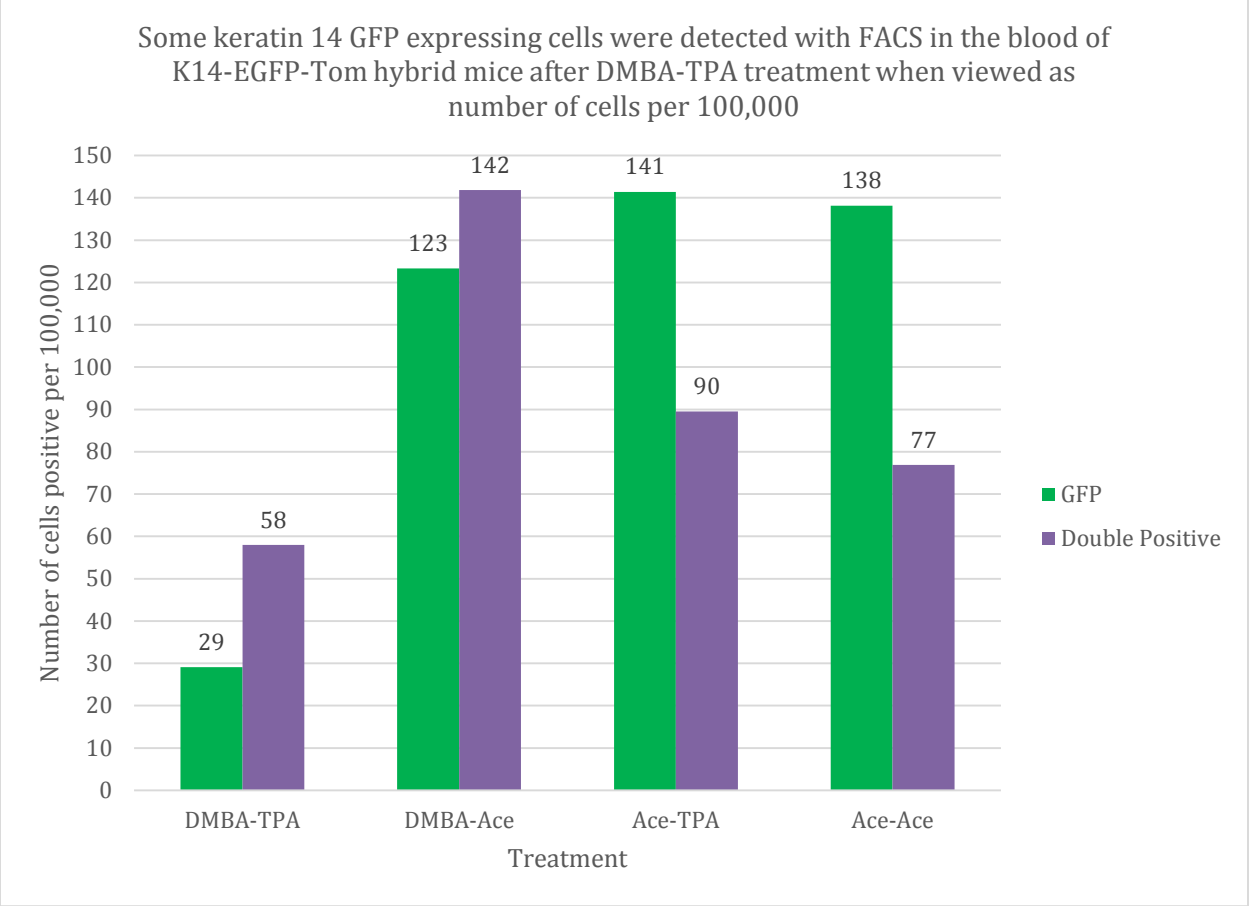


Figure 6. Fluorescent activated cell sorting (FACS) shows more K14-GFP expressing cells in the blood of K14-EGFP-Tom hybrid mice after receiving DMBA and TPA treatment than in the bone marrow. The number of positive K14-EGFP fluorescent labeled cells per 100,000 sorted in the blood of K14-EGFP-Tom hybrid mice after 1x initiation with DMBA and 6x promotion treatments with TPA. Acetone (Ace) was used as a control. DMBA and TPA treatments were also performed on K14Cre and B6 mice as negative controls (data omitted). In the K14-EGFP-Tom mice, K14 expressing cells naturally fluoresce “green” whereas all other cells naturally fluoresce “red”. When analyzing the blood samples by number of GFP positive or GFP/TomatoRed double positive cells, there are fewer GFP positive and GFP/TomatoRed double positive cells in the DMBA-TPA treatment groups than in the various controls. There are more double positive cells relative to GFP positive cells in the DMBA treatment groups than in the Acetone treatment groups.

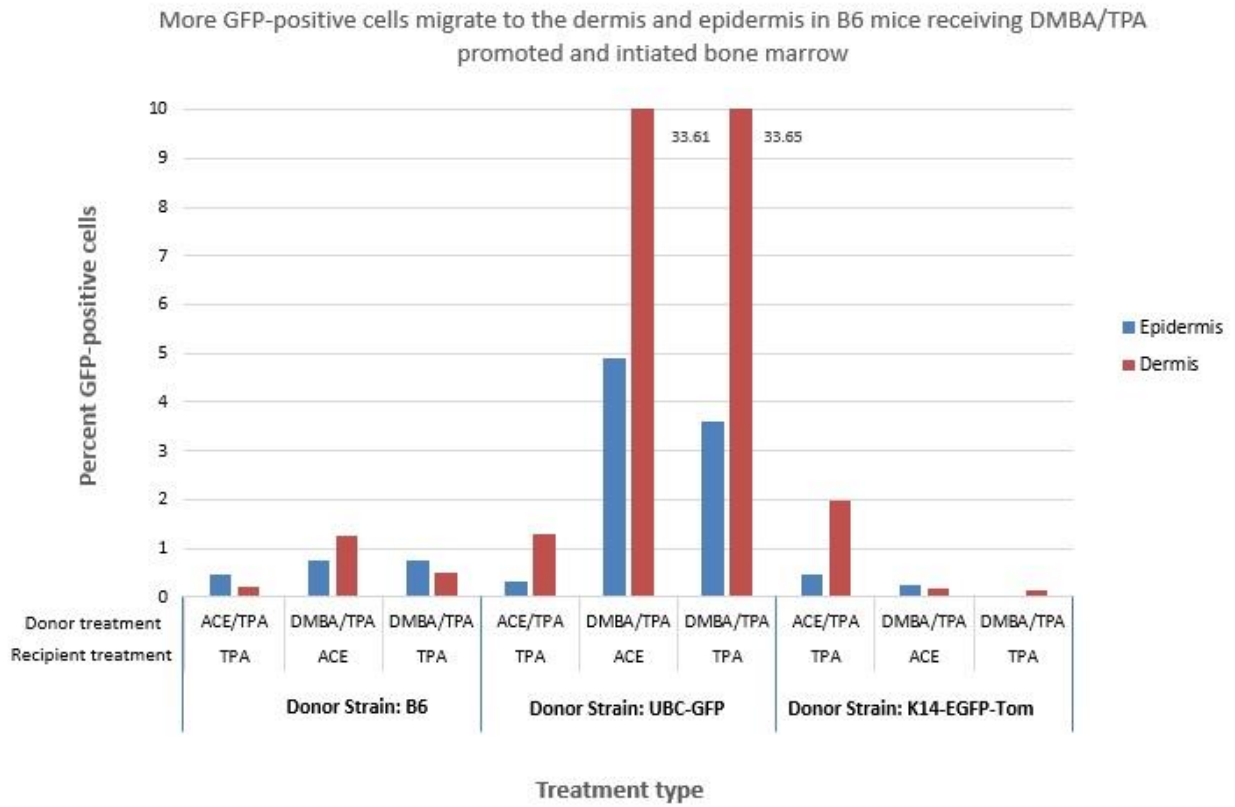


Figure 7a. Immunohistochemistry staining shows an increased number of GFP-positive bone marrow cells migrating to the skin relative to controls in B6 mice receiving DMBA/TPA promoted and initiated bone marrow. B6 bone marrow recipients were treated with either TPA or acetone (ACE) following transplants with bone marrow treated with DMBA and/or TPA. DMBA and TPA treatments were also performed on UBC-GFP and B6 mice as positive and negative controls, respectively. In the K14-EGFP-Tom mice, K14 expressing cells naturally fluoresce “green”. Skin samples from each treatment group (~1-3 mice per group) were processed and stained using standard IHC protocols. Images were taken of each sample and imported into ImageJ for counting. Positive GFP cells were counted as percent positive of the total number of cells (~1000-4000 per sample) in the dermis or epidermis. In K14-EGFP-Tom mice, there are few positive cells in mice receiving bone marrow treated with DMBA and TPA, however results may be skewed due to the small sample size requested for this study. Issues with mouse breeding have delayed our analysis efforts and therefore data are currently incomplete.

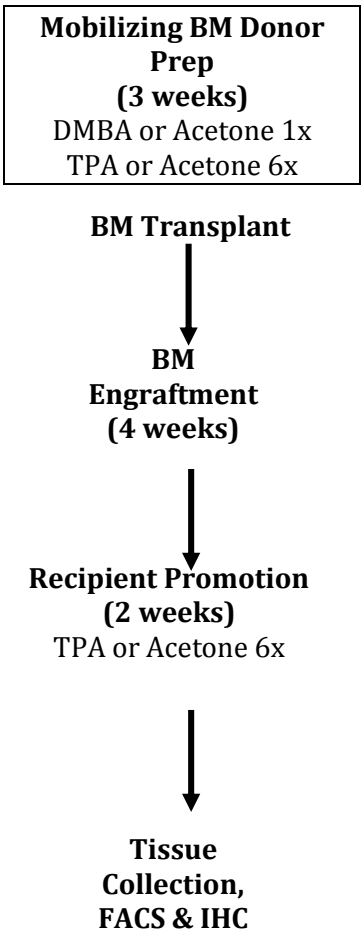


Figure 7b. Schematic of bone marrow transplant pilot study. Male C57Bl/6, UBC-GFP, and K14-EGFP-Tom mice were treated topically one time with either DMBA or acetone and then topically six times with TPA or acetone to mobilize bone marrow cells. After the three-week period of initiation and promotion, bone marrow was harvested from male donors and transplanted via tail vein injection to female C57Bl/6 recipient mice. The female recipients were allowed to rest for four weeks while the bone marrow engrafted then received six treatments of topical TPA or acetone over the course of two weeks. At the end of the nine week study, blood and bone marrow from the female recipients were analyzed by FACS and skin was collected for immunohistochemistry as shown in Figure 7a.

SUPPORTING DATA:

- Figures relevant to each Task are presented as Appendix pages of this Progress Report but are described following the presentation of each Task and the Methods used to achieve it.